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1. Your reference — HP/LP5911490

2. Patent applicant
0111872.8

15 MAY 2001

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*
Patents ADP number *(if you know it)*

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(SEE CONTINUATION SHEET)

If the applicant is a corporate body, give the country/state of its incorporation — GB

8145351.1

4. Title of the invention — THERAPEUTIC AGENTS AND METHODS

5. Name of your agent *(if you have one)* — MEWBURN ELLIS

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ADP No:	7984540 04
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Therapeutic Agents and Methods

The present invention relates to pharmaceutical compositions and compounds for the therapeutic delivery of carbon monoxide.

Carbon monoxide (CO) is, by common definition, a colorless, odorless, tasteless, non-corrosive gas of about the same density as that of air and is the most commonly encountered and pervasive poison in our environment. It is generally produced by the incomplete combustion of fossil fuels such as natural gas, propane, coal, gasoline and wood. In the atmosphere, the average global levels are estimated to be 0.19 parts per million (p.p.m.), 90% of which comes from natural sources including ocean micro-organism production, and 10% of which is generated by human activity. Thus, inhalation of even small quantities of CO is inevitable for living organisms.

Depending on the extent and time of exposure, CO is capable of producing a myriad of debilitating and harmful residual effects to the organism (1). The most immediate of these effects, and perhaps the most notorious one, is the binding to hemoglobin in the blood stream, which rapidly decreases the oxygen transport capability of the cardiovascular system. Paradoxically, more than half a century ago it was found that CO is constantly formed in humans in small quantities (2), and that under certain

pathophysiological conditions this endogenous production of CO may be considerably increased (3-5). The discovery that hemoglobin, a heme-dependent protein, is required as substrate for the production of CO *in vivo* (6,7) and the identification of the enzyme heme oxygenase as the crucial pathway for the generation of this gaseous molecule in mammals (8) set the basis for the early investigation of an unexpected and still unrecognized role of CO in the vasculature (9). The succeeding cloning (10) and characterization of constitutive (HO-2) and inducible (HO-1) isoforms of heme oxygenase (11-13) as well as studies on the kinetics and tissue distribution of these enzymes (14) started to reveal a major importance of this pathway in the physiological degradation of heme. That is, the end products of heme degradation (CO, biliverdin and bilirubin) might possess, after all, crucial biological activities (15-17).

With regard to the cardiovascular system, the recognition that CO possesses vasodilatory properties (18-20) is, perhaps, the most significant evidence in favor of a pharmacological function of CO. Although the molecular mechanisms and the chemical modifications that are required to transduce the signals mediated by CO into a specific biological effect need to be fully elucidated, convincing scientific reports have recently highlighted the signaling properties of endogenously generated CO (21-24).

Experimental studies on the physiological effects of nitric oxide (NO) have been facilitated by the development of a wide variety of organic compounds that spontaneously release NO and can be easily acquired to reproduce a physiological or pathophysiological function of NO. There is now abundant literature on the different types of NO donors and NO-releasing agents that, depending on their stability and half-life, can be used in disparate *in vitro* and *in vivo* models to simulate the biological activity of this important signaling molecule (25,26). In clinical practice, compounds that deliver NO into the circulation such as sodium nitroprusside and glyceryl trinitrate are used to lower blood pressure and treat certain cardiovascular diseases (27). Drugs containing a functional NO group that can selectively target an organ or tissue are currently being developed or are under clinical trials for the treatment of specific pathophysiological states (28,29). However, to date no compounds capable of delivering CO therapeutically have been identified.

US Patent 5,882,674 (Herrmann *et al.*) proposes administration of CO via transdermal delivery systems containing metal carbonyl complexes such as iron pentacarbonyl and iron enneacarbonyl. However, since this document provides no experimental data, and no description of specific devices, it is not clear how this proposal can be made to work. In particular it is not

stated whether the iron carbonyl complex is intended to be absorbed from the patch, to release CO within the body, or whether the complex breaks down within the patch to release CO which then enters the bloodstream after absorption through the skin.

As exemplified by the experimental data detailed below, the present inventors have found that metal carbonyl compounds can be used to deliver CO to a physiological target in a number of ways.

Accordingly the present invention provides a pharmaceutical composition, for delivery of carbon monoxide to a physiological target, comprising a metal carbonyl compound or pharmaceutically acceptable salt thereof and at least one pharmaceutically acceptable carrier, wherein the metal carbonyl makes available CO suitable for physiological effect by at least one of the following means:

1) CO derived by dissociation of the metal carbonyl is present in the composition in dissolved form;

2) on contact with a solvent the metal carbonyl releases CO;

3) on contact with a tissue, organ or cell the metal carbonyl releases CO;

4) on irradiation the metal carbonyl releases CO.

Certain metal carbonyl compounds are capable of releasing CO on contact with a suitable solvent. When the pharmaceutical composition is to be administered in

liquid form, this solvent may form a component part of the pharmaceutical composition. Thus in this aspect of the invention, the pharmaceutical composition contains CO derived from the metal carbonyl in dissolved form. The conditions under which the carbonyl compound is dissolved in the solvent during preparation of the pharmaceutical may be controlled such that the CO thus released is retained in solution. This may be facilitated where an equilibrium exists between the dissociated components and the undissociated carbonyl.

The dissociated components of the parent carbonyl may themselves be metal carbonyl complexes capable of releasing further CO. For example, when $[Ru(CO)_3Cl_2]_2$ is dissolved in DMSO, CO is liberated into solution, and di-carbonyl complexes are formed, which may themselves be capable of releasing further CO.

In a further aspect of the invention, the pharmaceutical composition may not itself contain dissolved CO, but may be prepared such as to release CO on contact with a suitable solvent or medium. For example, the composition may contain a metal carbonyl compound capable of releasing CO on contact with water, e.g. on contact with an aqueous physiological fluid, such as blood or lymph. Alternatively, the pharmaceutical composition may be intended to be dissolved in water prior to administration. Such pharmaceutical compositions may be prepared in solution or in solid

form, such as in tablet form. If they are in solution form, they will typically be prepared in a solvent which does not support dissociation of the metal carbonyl compound, such that release of CO takes place only on contact with the appropriate solvent.

In another aspect of the invention the pharmaceutical composition may contain a metal carbonyl compound which releases CO on contact with a tissue, organ or cell. It is shown below that certain metal carbonyl compounds do not release CO to solution but are nevertheless capable of releasing CO to physiological cellular materials or tissues, such as vascular endothelium. For example, $[Fe(SPh)_2(2,2'$-bipyridine$)(CO)_2]$ is shown below not to release CO to myoglobin in solution, but is nevertheless capable of promoting dilatation of pre-contracted aortic rings. Without wishing to be limited by any particular theory, it is thought that CO may be released from such compounds as a result of an oxidation-reduction reaction, mediated by cellular components such as cytochromes.

In a further aspect of the invention, the pharmaceutical composition may contain a metal carbonyl compound which releases CO on irradiation. The compound may be irradiated prior to administration, for example to produce a solution of dissolved CO, or may be irradiated *in situ* after administration. It is contemplated that such compositions may be used to provide controlled,

localised release of CO. For example a pharmaceutical composition of this type may be administered during surgery, and CO released specifically at a site in need thereof, e.g. to induce vasodilation, by localised irradiation by means of a laser or other radiant energy source, such as UV rays.

Typically the pharmaceutical compositions of the present invention release CO such as to make it available to a therapeutic target in dissolved form. However, in some circumstances CO may be released from a metal carbonyl directly to a non-solvent acceptor molecule.

It will be apparent that pharmaceutical compositions according to the present invention may be capable of delivering CO therapeutically through one or more of the above described modes of action.

Typically the metal carbonyl compound comprises a complex of a transition metal, preferably a transition metal from group VIIa or group VIII. Preferably, the carbonyl compound comprises a complex of at least one of Fe, Mn, Ru, Rh or Co, having at least one carbonyl ligand.

The metal carbonyl compounds may be regarded as complexes, because they comprise CO groups coordinated to a metal centre. However the metal may be bonded to other groups by other than coordination bonds, e.g. by ionic or covalent bonds. Thus groups other than CO which form part of the metal carbonyl compound need not strictly be

"ligands" in the sense of being coordinated to a metal centre via a lone electron pair, but will be referred to herein as "ligands" for ease of reference.

Thus, the ligands to the metal may all be carbonyl ligands, as e.g. in $[Mn_2(CO)_{10}]$. Alternatively, the carbonyl compound may comprise at least one modulatory ligand. By this is meant a ligand which is not CO, but which modulates a particular property of the complex, such as the tendency to release CO, solubility, hydrophobicity, stability, electrochemical potential, etc. Thus suitable choices of ligand may be made in order to modulate the behaviour of the compound. For example it may be desirable to modulate the solubility of the compound in organic and/or aqueous solvents, its ability to cross cell membranes, its rate of release of CO on contact with a particular solvent or cell type, or on irradiation, etc.

Such ligands are typically neutral or anionic ligands, such as halide, or derived from Lewis bases and having N, P, O, or S as the coordinating atom(s). Examples include, but are not limited to, sulfoxides such as dimethylsulfoxide, natural and synthetic amino acids and their salts for example, glycine, cysteine, and proline, amines such as NEt_3 and $H_2NCH_2CH_2NH_2$, aromatic bases and their analogues, for example, bi-2,2'-pyridyl, indole, pyrimidine and cytidine, drug molecules such as YC-1 (2-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole),

thiols and thiolates such as EtSH and PhSH, chloride, bromide and iodide, carboxylates such as formate, acetate, and oxalate, ethers such as Et_2O and tetrahydrofuran, alcohols such as EtOH, and nitriles such as MeCN.

CO is thought to act at least in part through the stimulation of guanylate cyclase activity. Thus the metal carbonyl compound may comprise ligands which modulate the effect of CO on guanylate cyclase. For example, the drug YC-1 (3-(5'-hydroxymethyl-2'-furyl)-1-benzylindole) is thought to enhance stimulation of guanylate cyclase by CO. Thus incorporation of ligands such as YC-1 or derivatives thereof into the metal carbonyl compounds can alter or enhance the biological effects of the released CO.

Thus the properties of pharmaceutical compositions of the present invention may be tailored as required by appropriate choice of metal centres and number and type of associated ligands in the metal carbonyl compound.

The metal carbonyl compound may further comprise a targeting moiety, to facilitate release of CO at an appropriate site. The targeting moiety is typically capable of binding a receptor on a particular target cell surface, in order to promote release of CO at the required site. The targeting moiety may be a part of a modulating ligand capable of binding to a receptor found on the surface of the target cells, or may be derived

from another molecule, such as an antibody directed against a particular receptor, joined to the complex by a suitable linker.

The present invention also provides a pharmaceutical composition for delivery of CO, comprising as active ingredient a compound of the formula $M(CO)_xA_y$ where x is at least one, y is at least one, M is a metal, A is an atom or group bonded to M by an ionic, covalent or coordination bond, and, in the case where y>1, each A may be the same or different, or a pharmaceutically acceptable salt of such a compound. Typically, M is a transition metal, particularly of group VIIa or group VIII, and A may be selected from halogens and groups having N, P, O or S atoms, providing lone electron pairs for coordination bonding to M.

The pharmaceutical compositions of the present invention typically comprise a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, subcutaneous, nasal, intramuscular, intraperitoneal, or suppository routes.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A

tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included. Pharmaceutically acceptable amounts of other solvents may also be included, in particular where they are required for dissolving the particular metal carbonyl compound contained in the composition.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will typically be in the form of a parenterally acceptable solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required. Delivery systems for needle-free injection are also known, and compositions for use with such systems may be prepared accordingly.

Administration is preferably in a prophylactically effective amount or a therapeutically effective amount (as the case may be, although prophylaxis may be

considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

When formulating pharmaceutical compositions according to the present invention, the toxicity of the active ingredient and/or the solvent must be considered. The balance between medical benefit and toxicity should be taken into account when determining The dosages and formulations of the compositions will typically be determined so that the medical benefit provided outweighs any risks due to the toxicity of the constituents.

There is further provided a method of introducing CO to a mammal comprising the step of administering a pharmaceutical composition according to the present invention. CO is thought to act at least in part through stimulation or activation of guanylate cyclase. CO is

thought to have functions as, inter alia, a neurotransmitter and a vasodilating agent. Accordingly there is provided a method of delivering CO to a mammal for stimulation of guanylate cyclase activity. There is further provided a method of delivering CO to a mammal for stimulating neurotransmission or vasodilation.

The heme oxygenase 1 (HO-1) pathway is thought to represent a pivotal endogenous inducible defensive system against stressful stimuli including UVA radiations, carcinogens, ischaemia-reperfusion damage, endotoxic shock and several other conditions characterised by production of oxygen free radicals (30-32). The protective effect of HO-1 is attributed to the generation of the powerful antioxidants biliverdin and bilirubin and the vasoactive gas CO. Expression of HO-1 has been linked with cardiac xenograft survival (33), suppression of transplant arteriosclerosis (34) and amelioration of post-ischemic myocardial dysfunction (35). HO-1 has also been directly implicated in the resolution phase of acute inflammation in rats (36). Other pathological situations, such as haemorrhagic shock in brain and liver as well as sepsis (37-39), are characterized by induction of the HO-1 gene, which seems to play a crucial role in counteracting the vascular dysfunction caused by these pathophysiological states. Increased generation of CO as a consequence of HO-1 induction markedly affects vessel contractility and diminishes acute hypertension in the

whole organism (23,40). Exposure of animals to ambient air containing low concentrations of CO or transfection of the HO-1 gene results in protection against hyperoxia-induced lung injury *in vivo*, a mechanism mediated by attenuation of both neutrophil inflammation and lung apoptosis (cell death) (41,42). Exogenous CO gas also has the ability to suppress pro-inflammatory cytokines and modulate the expression of the anti-inflammatory molecule, IL-10, both *in vitro* and *in vivo* (43). Therefore administration of CO in accordance with the invention may be used for treatment of any of these conditions, for modulation of inflammatory states and regression of other pathophysiological conditions including cancer.

Accordingly there is provided a method of introducing CO to a mammal comprising the step of administering a pharmaceutical composition according to the present invention, for treatment of acute and chronic hypertension, radiation damage, endotoxic shock, inflammatory-related diseases such as asthma and rheumatoid arthritis, hyperoxia-induced injury, apoptosis, cancer, transplant rejection, arteriosclerosis, post-ischemic organ damage, myocardial infarction and angina, haemorrhagic shock or sepsis, penile erectile dysfunction, and adult respiratory distress syndrome.

The present invention also provides the use of a metal carbonyl compound as herein described in the manufacture of a medicament for stimulating neurotransmission or vasodilation, or for treatment of any of acute and chronic hypertension, radiation damage, endotoxic shock, inflammatory-related diseases such as asthma and rheumatoid arthritis, hyperoxia-induced injury, apoptosis, cancer, transplant rejection, arteriosclerosis, post-ischemic organ damage, myocardial infarction and angina, haemorrhagic shock or sepsis, penile erectile dysfunction, and adult respiratory distress syndrome. Such medicaments may be adapted for administration by an oral, intravenous, subcutaneous, nasal, inhalatory, intramuscular, intraperitoneal or suppository route.

The present invention also provides a compound of the formula $M(CO)_xA_y$ where x is at least one, y is at least one, M is a metal, A is an atom or group bonded to M by an ionic, covalent or coordination bond, and where y>1 each A may be the same or different, or a pharmaceutically acceptable salt of such a compound, for use in a method of medical treatment. Examples of such compounds include $[Ru(CO)_3Cl_2]_2$, $[Ru(CO)_2(DMSO)_2Cl_2]$, $[Ru(CO)_3Cl_2(cytosine)]$, $[Ru(CO)_3(glycinate)Cl]$, $[Fe(SPh)_2(2,2'\text{-bipyridine})(CO)_2]$, and $[Fe(SPh)_2(NH_2CH_2CH_2NH_2)(CO)_2]$.

It is further considered that, in place of the metal carbonyl compounds discussed above, the pharmaceutical compositions of the present invention may comprise oxalate compounds, formic acid, or formate compounds, which may likewise deliver CO to a physiological target. For example, bis-(2,4-dinitrophenyl) oxalate is known to decompose in water to liberate CO into solution. Therefore the present invention further provides a pharmaceutical composition, for delivery of carbon monoxide to a physiological target, comprising formic acid, a formate, or an oxalate, or a pharmaceutically acceptable salt thereof and at least one pharmaceutically acceptable carrier, wherein the formic acid, formate, or oxalate makes available CO suitable for physiological effect.

It is thought that the nitrophenyl groups of bis-(2,4-dinitrophenyl) oxalate are good leaving groups, because of the electron-withdrawing effects of the nitro groups, and that this may promote the decomposition of the oxalate to yield CO.

It is therefore considered that oxalates or formates having in which the acid groups are linked, e.g. by an ester bond, to aromatic groups with electron-withdrawing substituents, such as tosyl groups, are particularly suitable for use in pharmaceutical compositions according to the present invention.

There is further provided a method of introducing carbon monoxide to a mammal comprising the step of administering a pharmaceutical composition comprising formic acid, a formate, or an oxalate, or a pharmaceutically acceptable salt thereof, and at least one pharmaceutically acceptable carrier.

All the above discussion and disclosure relating to metal carbonyl compounds is also considered to relate to formic acid, formates and oxalates.

Throughout this application, references to medical treatment are intended to include both human and veterinary treatment, and references to pharmaceutical compositions are accordingly intended to encompass compositions for use in human or veterinary treatment.

Experimental data illustrating the present invention will now be described by reference to the accompanying figures, in which:

Figure 1 shows apparatus for measuring release of CO by metal carbonyl complexes on irradiation and structures of $[Mn_2(CO)_{10}]$ and $[Fe(CO)_5]$.

Figure 2 shows myoglobin absorption spectra.

Figure 3 shows NMR spectra illustrating the dissolution of $[Ru(CO)_3Cl_2]_2$ in DMSO.

Figure 4 shows viability data for cells treated with metal carbonyl compounds.

Figure 5 shows relaxation of aortic rings on treatment with metal carbonyl complexes.

Figure 6 shows the effects of various treatments on perfused rat hearts.

Figure 7 shows expression of heme oxygenase 1 in rat hearts.

Figure 8 shows the effects of various treatments on rat mean arterial pressure.

For these experiments, iron pentacarbonyl, $[Fe(CO)_5]$, dimanganese decacarbonyl, $[Mn_2(CO)_{10}]$, tricarbonyldichlororuthenium (II) dimer, $[Ru(CO)_3Cl_2]_2$, and ruthenium(III) chloride hydrate, $RuCl_3$, were purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK). Stock solutions of metal carbonyl complexes were prepared fresh prior to each experiment by dissolving the compounds in dimethyl sulfoxide (DMSO). Hemin (ferriprotoporphyrin IX chloride) and tin protoporphyrin IX (SnPPIX) were from Porphyrin Products Inc. (Logan, Utah, USA). Stock solutions of both porphyrins were prepared by dissolving the compounds in 0.1 M NaOH and then adjusting the pH to 7.4 by addition of 0.01 M phosphate buffer. The guanylate cyclase inhibitor, [1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one] (ODQ), was obtained from Alexis Corporation (Bingham, Nottingham, UK) and polyclonal rabbit anti-HO-1 antibodies were purchased from Stressgen (Victoria, Canada). Horse heart myoglobin, N^G-nitro-L-arginine methyl esther (L-NAME) and all other reagents were from Sigma, unless otherwise specified.

All data are expressed as mean ± s.e.m. Differences between the groups analysed were assessed by the Student's two-tailed *t*-test, and an analysis of variance (ANOVA) was performed where more than two treatments were compared. Results were considered statistically significant at $P<0.05$.

<u>Detection of CO liberated from transition metal carbonyl complexes.</u>

The release of CO from metal carbonyl complexes was assessed spectrophotometrically by measuring the conversion of deoxymyoglobin (deoxy-Mb) to carbonmonoxy myoglobin (MbCO). MbCO has a distinctive absorption spectrum between 500 and 600 nm, and changes at 540 nm were used to quantify the amount of CO liberated. Myoglobin solutions (66 μM final concentration) were prepared freshly by dissolving the protein in 0.04 M phosphate buffer (pH 6.8). Sodium dithionite (0.1 %) was added to convert myoglobin to deoxy-Mb prior to each reading. All the spectra were measured using a Helios α spectrophotometer.

Direct addition of iron pentacarbonyl, $[Fe(CO)_5]$, or dimanganese decacarbonyl, $[Mn_2(CO)_{10}]$, to myoglobin solutions did not result in any appreciable formation of carbonmonoxy myoglobin (MbCO) over time (data not shown). This is consistent with the notion that these two transition metal carbonyl complexes do not release CO

unless stimulated by light (44,45). Therefore release of CO was induced by exposing these metal carbonyl complexes to a cold light source and allowing the gas to diffuse through a membrane before reacting with myoglobin as shown in Figure 1.

Five hundred microliters of iron pentacarbonyl ($[Fe(CO)_5]$, 99.9%) or 1 ml of dimanganese decacarbonyl ($[Mn_2(CO)_{10}]$, 13 mM in DMSO) (see also chemical structure) were placed in a plastic tube. A cell culture insert (Costar) was sealed on top in order to create two separate chambers with a 0.6 cm air space between the solution and the insert membrane (0.4 μm). One and a half milliliters of deoxy-Mb solution (66 μM) was placed in the insert which was finally covered with parafilm. The carbonyl solution was then exposed to cold light to stimulate CO release, allowing the gas to diffuse through the membrane into the myoglobin solution. Aliquots of the myoglobin solution were taken at different times and the conversion of deoxy-Mb to MbCO measured spectrophotometrically.

The spectral change on transition from deoxy-Mb to MbCO was measured by bubbling CO gas to a solution of deoxy-Mb (Figure 2a). Upon illumination, $[Fe(CO)_5]$ and $[Mn_2(CO)_{10}]$ produced a similar change in the absorbance spectrum of myoglobin, with a gradual increase in MbCO formation observed over time; in both cases the distinctive identified spectra were the ones typical of

MbCO (Figures 2b and 2c). Under the experimental conditions used, a complete saturation of the myoglobin solution was achieved by $[Mn_2(CO)_{10}]$ (13 μmol/ml) in approximately 40 min of continuous exposure to light (Figure 2d).

Various metal carbonyl complexes were tested for their ability to elicit MbCO formation when added directly to a deoxy-Mb solution. To different extents, $[Ru(CO)_3Cl_2]_2$, $[Ru(CO)_2(DMSO)_2Cl_2]$, $[Ru(CO)_3Cl_2(cytosine)]$ and $[Ru(CO)_3(glycinate)Cl]$ all released CO when added directly to the Mb solution whereas no MbCO was detected in the case of $[Fe(SPh)_2(2,2'\text{-bipyridine})(CO)_2]$ and $[Fe(SPh)_2(H_2NCH_2CH_2NH_2)(CO)_2]$.

Data for the tricarbonyldichlororuthenium (II) dimer $[Ru(CO)_3Cl_2]_2$ are shown in Figure 2e. The metal carbonyl complex was solubilized in DMSO (9.7 mM stock solution), aliquots of 2 to 32 μl were added directly to 1 ml of deoxy-Mb solutions (66 μM) and absorption spectrum determined immediately after mixing the samples by inversion. A linear regression analysis of the saturation curve of MbCO revealed that for each mole of $[Ru(CO)_3Cl_2]_2$ approximately 0.7 moles of CO are liberated (Figure 2f).

<u>NMR studies of $[Ru(CO)_3Cl_2]_2$</u>

Further studies were conducted on the chemistry of transition metal carbonyls using NMR spectroscopy. The

^{13}C NMR spectrum showed that $[Ru(CO)_3Cl_2]_2$ freshly dissolved in DMSO does not exist as a dimer; in fact, two distinct peaks corresponding to tri-carbonyl (1) and di-carbonyl (2) monomers could be identified. The NMR analysis reveals that, during the solubilization process, DMSO acts as a coordinated ligand to ruthenium thereby promoting the formation of the monomers.

Figure 3a shows a 100.62 MHz $^{13}C\{^1H\}$ NMR spectrum taken during the first 23 min of the reaction between freshly prepared $[RuCl_2(CO)_3]_2$ and d_6-DMSO. The solution very slowly produced fine bubbles of a gas, presumably CO, implied by the formation of 2. When the experiment was repeated by dissolving initially the metal complex in DMSO and then diluting with $CDCl_3$, the assignment of the signals coincided with the published ^{13}C chemical shifts of fac-$[RuCl_2(CO)_3(DMSO)]$ (1, δ 183.0, 186.8), cis, cis, trans-$[RuCl_2(CO)_2(DMSO)_2]$ (2, δ 185.0) and cis, cis, cis-$[RuCl_2(CO)_2(DMSO)_2]$ (3, δ 186.0, 191.9) (46). Figure 3b shows a 100.62 MHz $^{13}C\{^1H\}$ NMR spectrum taken after $[RuCl_2(CO)_3]_2$ in d_6-DMSO was warmed at 50 °C for 5 min and left to accumulate overnight. In addition to the peaks that could be assigned to 1, 2 and 3, there are carbonyl signals at δ 187.9 and 190.5 due to unidentified species.

The detection of di-carbonyl monomers demonstrates that CO is liberated; the ^{13}C NMR spectrum also suggests that the ratio between 1 and 2 is 40:60.

Effect of $[Ru(CO)_3Cl_2]_2$ on cell viability

As there are no precedent studies on the use of metal carbonyl complexes in biological systems, it was necessary to evaluate the potential cytotoxic effect of these compounds. Therefore, the viability of cells in culture was determined after short or prolonged exposure to various concentrations of metal carbonyls.

Rat vascular smooth muscle cells were obtained from the Coriell Cell Repository (Camden, NJ, USA) and grown in Dulbecco's Minimal Essential Medium (MEM) supplemented with 20% foetal calf serum, 2 x MEM vitamins, 2 x MEM non-essential and essential amino acids, penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Confluent cells were treated with different concentrations of metal carbonyl for various times and cell viability was assessed using a colorimetric assay kit from Promega (Madison, WI, USA) as previously described (47) after 3 or 24 h incubation, or after 3 h exposure to the agents followed by 21 h incubation in complete media. Results are expressed as the mean ± s.e.m. of 6 independent experiments and differences were considered statistically significant at $P < 0.05$ (*).

Exposure of $[Fe(CO)_5]$ to light gradually resulted in deposition of a green-brown precipitate, and so viability studies on this metal carbonyl were not pursued. Nevertheless, $[Fe(SPh)_2(2,2'\text{-bipyridine})(CO)_2]$ proved to elicit a marked vasodilatatory effect (see below).

Therefore the synthesis of metal carbonyls containing iron as transition metal that are more compatible with biological tissues should be kept into consideration for future experiments as dinitrosyl iron complexes are currently investigated as potential carriers of NO (48).

As shown in Figure 4b, treatment of vascular smooth muscle cells for 3 h with $[Ru(CO)_3Cl_2]_2$ (0-420 μM final concentration) did not promote any detectable cytotoxicity. Similarly, cell viability was well preserved after exposure to this metal carbonyl for 3 h followed by an additional 21 h incubation in complete medium. A pronounced cytotoxic effect (>50% loss in cell viability) was only apparent after prolonged exposure (24 h) to very high concentrations (> 400 μM) of $[Ru(CO)_3Cl_2]_2$.

Treatment of cells with the same amounts of vehicle (DMSO) or equivalent molar concentrations of ruthenium ($RuCl_3$) did not cause any appreciable decrease in cell viability over time (Figure 4a and 4c, respectively) indicating that neither the vehicle nor the metal are responsible for the observed cytotoxic effect of $[Ru(CO)_3Cl_2]_2$.

In the case of $[Mn_2(CO)_{10}]$ (0-100 μM), no major cytotoxicity on smooth muscle cells was detected after exposure for 24 h in complete medium (data not shown).

Vasodilatory effect of CO released from $[Ru(CO)_3Cl_2]_2$

It has previously been demonstrated that increased endogenous CO as a result of HO-1 induction in rat aortas markedly attenuates vasoconstriction (23). To investigate whether CO released from metal carbonyl complexes evokes specific biological activities, we first assessed the effect of these complexes on vessel contractility using the isolated aortic ring model.

Transverse ring sections of thoracic aorta were isolated from male Lewis rats and suspended under a 2 g tension in an organ bath containing oxygenated Krebs-Henseleit buffer at 37 °C as previously described (23). The relaxation response to cumulative doses of metal carbonyl was assessed in aortic rings pre-contracted with phenylephrine (3 μM). Control rings were similarly treated by adding equal doses of DMSO (vehicle) to the organ bath. Results are shown in Table 1 and Figure 5.

As shown in Figure 5, consecutive additions of $[Ru(CO)_3Cl_2]_2$ (222μM) to aortic rings pre-contracted with phenylephrine elicited a rapid and significant vasodilatation ($P<0.05$); the extent of relaxation was already pronounced after the first addition of the compound (45% more than control). Interestingly, after extensive washing, the phenylephrine-induced contraction was completely restored in control but not in $[Ru(CO)_3Cl_2]_2$-treated vessels indicating that this compound produces long-lasting effects.

The vasodilatory response mediated by metal carbonyls was almost totally abolished when reduced Mb (150μM), which avidly binds CO, was added to the buffer. Collectively, these findings are consistent with the fact that CO released from metal carbonyls possesses vasoactive properties.

As shown in Table 1, $[Ru(CO)_2(DMSO)_2Cl_2]$ also produced vasodilatation although the effect was less pronounced compared to $[Ru(CO)_3Cl_2]_2$. Interestingly, while $[Ru(CO)_3Cl_2(cytosine)]$ did not have any effect, $[Ru(CO)_3(glycinate)Cl]$ elicited significant vasodilatation which is consistent with the high release of CO detected with the MbCO assay. Notably, $[Fe(SPh)_2(2,2'$-bipyridine$)(CO)_2]$ which did not release any detectable CO to myoglobin, was still very effective in promoting vasorelaxation. On the other hand, the effect of $[Fe(SPh)_2(H_2NCH_2CH_2NH_2)(CO)_2]$. was less evident.

Table 1

Treatment	% Relaxation		
	1st addition	*2nd addition*	*3rd addition*
Vehicle (DMSO)	5.7±0.9	11.4±1.1	18.1±2.5
$[Ru(CO)_3Cl_2]_2$	49.9±2.7*	66.2±3.2*	74.1±4.1*
$[Ru(CO)_3Cl_2]_2$ + Mb	4.0±0.9†	8.6±0.4†	15.5±0.4†
$[Ru(CO)_3Cl_2]_2$ + ODQ	7.1±1.1†	23.6±3.8*†	55.5±6.9*†
$[Ru(CO)_2(DMSO)_2Cl_2]$	1.6	16	35
$[Ru(CO)_3Cl_2$(cytosine)]	3.2	10.3	12.6
$[Ru(CO)_3$(glycinate)Cl]	36	66.6	68.3
$[Fe(SPh)_2$(2,2'-bipyridine)$(CO)_2]$	50.8	60.5	75
$[Fe(SPh)_2(H_2NCH_2CH_2NH_2)(CO)_2]$	11	24.6	29.3

* $P < 0.01$, compared to vehicle; † $P < 0.01$ compared to $[Ru(CO)_3Cl_2]_2$.

Because CO is thought to modulate signal transduction mechanisms via increased production of cGMP, we investigated the effect of a selective inhibitor of guanylate cyclase (ODQ, 10 μM) on vessel contractility. As expected, ODQ considerably reduced the vasodilatation observed after the first two additions of $[Ru(CO)_3Cl_2]_2$;

however, it is of interest that the third addition of $[Ru(CO)_3Cl_2]_2$ still elicited a substantial vasodilatory action despite the presence of ODQ. Thus, the guanylate cyclase-cGMP pathway appears to be involved in the relaxation caused by this metal carbonyl complex.

Attenuation of vasoconstriction by metal carbonyls in perfused heart

Additional experiments were conducted to determine the biological activity of metal carbonyls on vascular function *in vivo* and compare it with HO-1-derived CO, by monitoring their effects on changes in coronary perfusion pressure (CPP) of isolated rat hearts.

Langendorff heart preparations were performed using male Lewis rats (300-350 g) as previously described by our group (35). Hearts were excised, the aorta cannulated and retrograde perfusion was established at a constant flow of 15 ml/min using Krebs-Henseleit buffer (in mM: 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.66 $MgSO_4$, 24.9 $NaHCO_3$, 1.18 KH_2PO_4, 5.55 glucose, 2.00 sodium pyruvate, 0.5 EGTA) bubbled with 95% O_2 and 5% CO_2 at 37°C (pH 7.4). Coronary perfusion pressure (CPP), a parameter indicative of coronary vessel contractility, was continuously measured by a pressure transducer connected to the aortic cannula and data analyzed with an Acknowledge software (BIOPAC System Inc.).

Hearts removed either from control rats (vehicle-treated) or from animals that were pre-treated with the heme oxygenase-1 inducer hemin (75 μmol/kg, i.p.) the day before, were initially equilibrated for 20 min on the Langendorff apparatus and then perfused with L-NAME (25 μM final concentration) to elicit vasoconstriction. The extent of CPP increase by L-NAME was also monitored over time in hemin-treated animals that received a heme oxygenase inhibitor (SnPPIX, 40 μmol/kg) 1 h prior to heart excision and in control hearts that were perfused with buffer supplemented with $[Mn_2(CO)_{10}]$ (13 μM final concentration). Since $[Mn_2(CO)_{10}]$ releases CO only by photodissociation, Krebs buffer containing $[Mn_2(CO)_{10}]$ was exposed to a cold light source immediately before entering the aortic cannula.

Vasoconstriction was elicited by perfusion with L-NAME and the extent of CPP increase measured over time. As shown in Figure 6, L-NAME caused a time-dependent increase in CPP, which reached a maximum (3-fold) after 30 min. Notably, perfusion of hearts with light-stimulated $[Mn_2(CO)_{10}]$ (13 μM) significantly delayed vasoconstriction and maintained CPP at much lower levels at the end of perfusion. When the buffer containing $[Mn_2(CO)_{10}]$ was not exposed to light, thus omitting the CO-induced release process, the extent of constriction mediated by L-NAME was unaffected (data not shown); in addition, perfusion with manganese chloride (negative

control) had no effect on myocardial CPP (data not shown).

The effect observed with $[Mn_2(CO)_{10}]$ could be similarly reproduced by induction of HO-1 in heart tissue by pretreatment with hemin. It has previously been reported that treatment of rats with hemin results in increased production of cardiac bilirubin, which is equimolar to endogenously generated CO (35). The rise in CPP mediated by L-NAME in hemin-treated hearts was markedly attenuated ($P<0.05$), to an extent similar to that produced by $[Mn_2(CO)_{10}]$ (Figure 6); predictably, the effect of hemin was completely reversed by tin protoporphyrin IX (SnPPIX), a heme oxygenase inhibitor. Thus, the vasoactive properties of the HO-1/CO pathway can be simulated by $[Mn_2(CO)_{10}]$.

Results are means ± s.e.m. of 6 independent experiments. * $P<0.05$ vs. vehicle-treated group (control).

<u>Expression of heme oxygenase in perfused rat hearts</u>

For immunohistochemistry analysis, sections of heart muscles (5 μm thickness) were treated with 0.3% H_2O_2 in methanol to block endogenous peroxidase activity. Immunohistochemical staining was performed using rabbit polyclonal antibody against HO-1 (1:1000 dilution) as previously described (23). The presence of HO-1 was indicated by the development of a brown color. For

Northern blot analysis, cardiac tissue was ground in a mortar under liquid nitrogen and suspended in guanidinium thiocyanate lysis buffer. Total RNA was then extracted using a modification of the method described by Chomczynski and Sacchi (49). RNA was run on a 1.3% denaturing agarose gel containing 2.2 M formaldehyde and transferred onto a nylon membrane overnight. The membrane was hybridized using [α-^{32}P]dCTP-labelled cDNA probes to rat HO-1 and GAPDH genes and bands analyzed using a densitometer as previously described (23,50).

Hearts were removed from Lewis rats 24 h after treatment with vehicle (control) or hemin (75 μmol/kg, i.p.) and immunostaining for HO-1 was assessed. For Northern blot analysis, rats were treated with hemin (75 μmol/kg, i.p.) and hearts removed at different time points to assess HO-1 mRNA levels (+ve, positive control).

Figure 7 confirms that HO-1 protein (7a) and mRNA (7b) are highly expressed in hearts 24 h after hemin treatment; interestingly, the immunostaining for HO-1 protein was primarily confined to the vessels of cardiac muscle (Figure 7a, right panel).

Animal studies

Since it has previously been reported that HO-1-derived CO also prevents acute hypertension *in vivo* (40),

experiments were performed to examine the effectiveness of metal carbonyls in regulating mean arterial pressure in animals.

Lewis rats (280-350 g) were anaesthetised by intramuscular injection of 1 ml/kg Hypnorm (fentanyl 0.315 mg/ml and fluanisone 10 mg/ml) followed 5 min later by an intraperitoneal injection of 5 mg/kg diazepam. Specially designed femoral artery and venous catheters were then surgically implanted as previously described (40). The arterial cannula was connected to a Grass pressure transducer and blood pressure monitored continuously using a polygraph recorder. Experiments were conducted on anaesthetized animals and recordings were made within 30 min of the surgical procedure.

Control rats (vehicle-treated) and animals that were pre-treated with hemin (75 μmol/kg, i.p) 24 h prior to blood pressure monitoring were then administered with an intravenous injection of 30 μmol/Kg L-NAME to elicit an increase in mean arterial pressure. The extent of blood pressure increase by L-NAME was also monitored over time in hemin-treated animals that received SnPPIX (40 μmol/kg, i.p.) and in control rats previously injected with $[Ru(CO)_3Cl_2]_2$ (60 μmol/kg, i.v.). In these two groups, SnPPIX or $[Ru(CO)_3Cl_2]_2$ were administered to animals 1 h prior to L-NAME injection.

Intravenous infusion of L-NAME in rats produced a rapid and significant increase in blood pressure

($P<0.05$); this effect was markedly suppressed by pre-treatment of animals with a single infusion of $[Ru(CO)_3Cl_2]_2$ prior to L-NAME administration (Figure 8). Moreover, and in analogy with the data on coronary vasoconstriction in isolated hearts, treatment of animals with hemin resulted in a significant suppression of the L-NAME-mediated hypertensive responses, which once again was totally reversed by blockade of the heme oxygenase pathway with SnPPIX (Figure 6d). Results are the means ± s.e.m. of 5 independent experiments. *$P<0.05$ vs. vehicle-treated group (control). Collectively, these *in vivo* findings attest a consistent and reproducible biological activity of metal carbonyls through their ability to carry and deliver CO.

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CLAIMS:

1. A pharmaceutical composition, for delivery of carbon monoxide to a physiological target, comprising a metal carbonyl compound or pharmaceutically acceptable salt thereof and at least one pharmaceutically acceptable carrier, wherein the metal carbonyl makes available CO suitable for physiological effect by at least one of the following means:

1) CO derived by dissociation of the metal carbonyl is present in the composition in dissolved form;

2) on contact with a solvent the metal carbonyl releases CO;

3) on contact with a tissue, organ or cell the metal carbonyl releases CO;

4) on irradiation the metal carbonyl releases CO.

2. A pharmaceutical composition according to claim 1, wherein the metal carbonyl compound is a complex of at least one of Fe, Mn, Ru, Rh or Co with at least one carbonyl ligand.

3. A pharmaceutical composition according to claim 1 or claim 2, wherein the metal is bound to at least one group other than CO.

4. A pharmaceutical composition according to claim 3, wherein the group other than CO is a modulatory group, which modulates the solubility of the compound and/or the release of CO from the compound.

5. A pharmaceutical composition according to any one of claims 1 to 4 adapted for administration by an oral, intravenous, subcutaneous, nasal, inhalatory, intramuscular, intraperitoneal or suppository route.

6. A pharmaceutical composition for delivery of CO, comprising as active ingredient a compound of the formula $M(CO)_xA_y$ where x is at least one, y is at least one, M is a metal, A is an atom or group bonded to M by an ionic, covalent or coordination bond, and in the case where y>1 each A may be the same or different, or a pharmaceutically acceptable salt of such a compound.

7. A pharmaceutical composition according to claim 6 wherein M is a transition metal.

8. A pharmaceutical composition according to claim 6 or claim 7, wherein A is selected from halogens and groups having N, P, O or S atoms providing lone electron pairs for coordination bonding to M.

9. A method of introducing CO to a mammal comprising the step of administering a pharmaceutical composition according to any one of claims 1 to 8.

10. A method according to claim 9, for the stimulation of guanylate cyclase activity.

11. A method according to claim 9 or claim 10, for stimulating neurotransmission or vasodilation, or for the treatment of any of acute and chronic hypertension, radiation damage, endotoxic shock, inflammatory-related diseases such as asthma and rheumatoid arthritis, hyperoxia-induced injury, apoptosis, cancer, transplant rejection, arteriosclerosis, post-ischemic organ damage, myocardial infarction and angina, haemorrhagic shock or sepsis, penile erectile dysfunction, and adult respiratory distress syndrome.

12. Use of a metal carbonyl compound in the manufacture of a medicament for administration by oral, intravenous, subcutaneous, nasal, inhalatory, intramuscular, intraperitoneal, or suppository routes, for the stimulation of neurotransmission or vasodilation, or for the treatment of any of hypertension, radiation damage, endotoxic shock, inflammation, hyperoxia-induced injury, apoptosis, cancer, transplant rejection,

arteriosclerosis, post-ischemic myocardial infarction, haemorrhagic shock, and sepsis.

13. Use according to claim 12 wherein the metal carbonyl compound is a complex of at least one of Fe, Mn, Ru or Co with at least one carbonyl ligand.

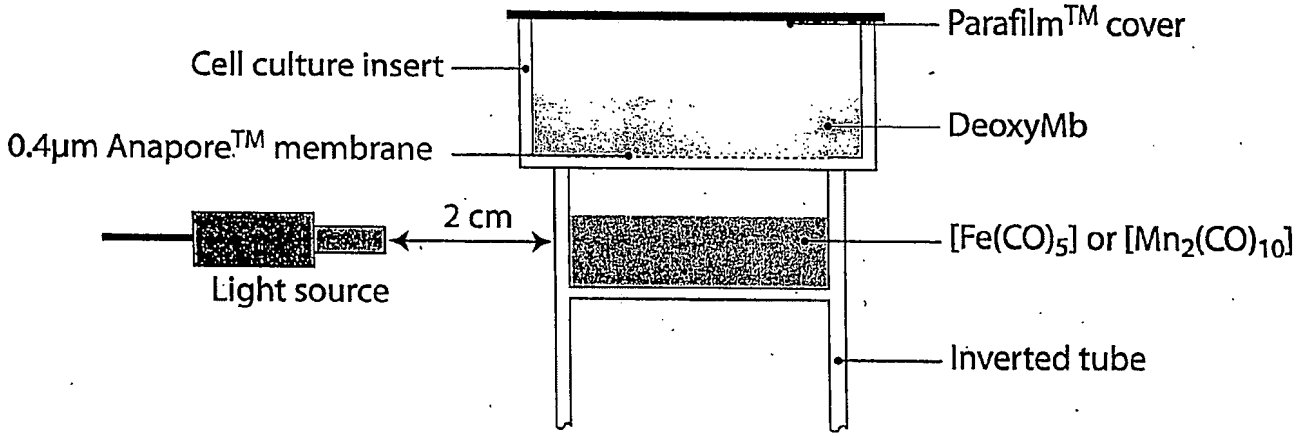
14. Use of a metal carbonyl compound as set out in any one of claims 6 to 8 in the manufacture of a medicament for the stimulation of neurotransmission or vasodilation, or for the treatment of any of acute and chronic hypertension, radiation damage, endotoxic shock, inflammatory-related diseases such as asthma and rheumatoid arthritis, hyperoxia-induced injury, apoptosis, cancer, transplant rejection, arteriosclerosis, post-ischemic organ damage, myocardial infarction and angina, haemorrhagic shock or sepsis, penile erectile dysfunction, and adult respiratory distress syndrome.

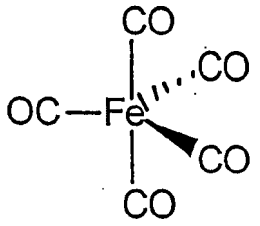
15. A compound of the formula $M(CO)_xA_y$ where x is at least one, y is at least one, M is a metal, A is an atom or group bonded to M by an ionic, covalent or coordination bond, and where y>1 each A may be the same or different, or a pharmaceutically acceptable salt of such a compound, for use in a method of medical treatment.

16. A pharmaceutical composition, for delivery of carbon monoxide to a physiological target, comprising formic acid, a formate, or an oxalate, or a pharmaceutically acceptable salt thereof, and at least one pharmaceutically acceptable carrier, wherein the formic acid, formate, or oxalate makes available CO suitable for physiological effect.

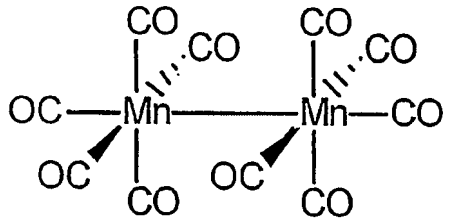
17. A method of introducing CO to a mammal comprising the step of administering a pharmaceutical composition according to claim 16.

18. A method according to claim 17 for stimulating guanylate cyclase activity, neurotransmission or vasodilation, or for the treatment of any of acute and chronic hypertension, radiation damage, endotoxic shock, inflammatory-related diseases such as asthma and rheumatoid arthritis, hyperoxia-induced injury, apoptosis, cancer, transplant rejection, arteriosclerosis, post-ischemic organ damage, myocardial infarction and angina, haemorrhagic shock or sepsis, penile erectile dysfunction, and adult respiratory distress syndrome.





Iron pentacarbonyl
$[Fe(CO)_5]$



Dimanganese decacarbonyl
$[Mn_2(CO)_{10}]$

Fig. 1

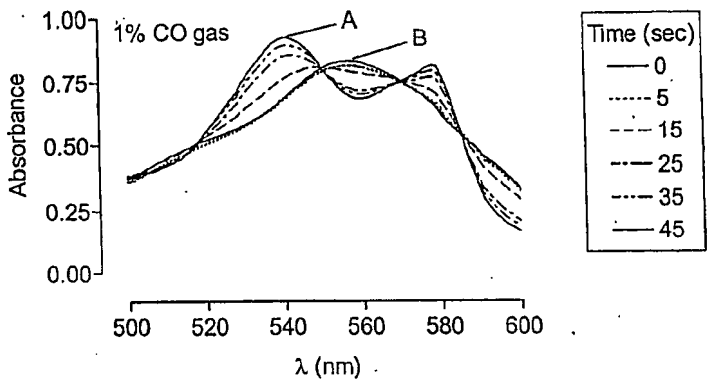


Fig. 2a

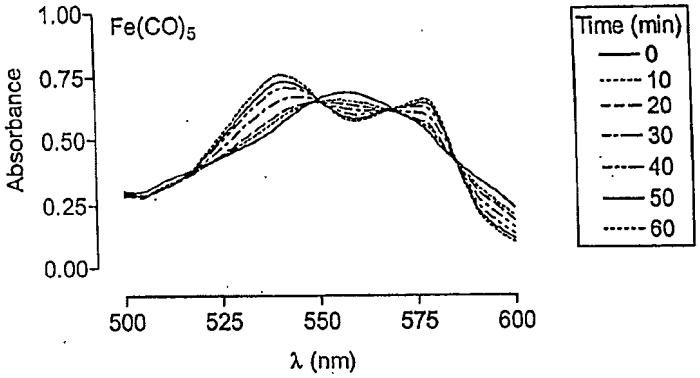


Fig. 2b

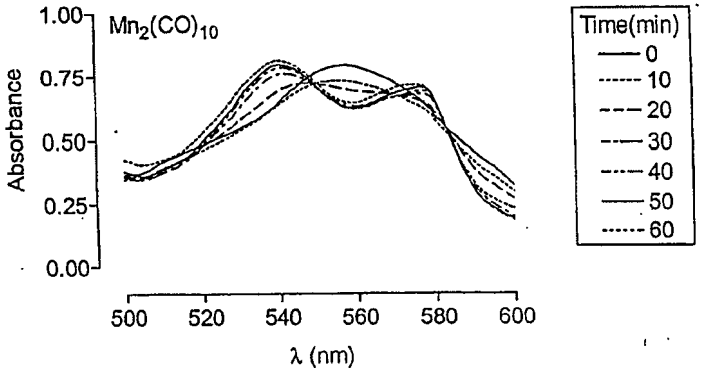
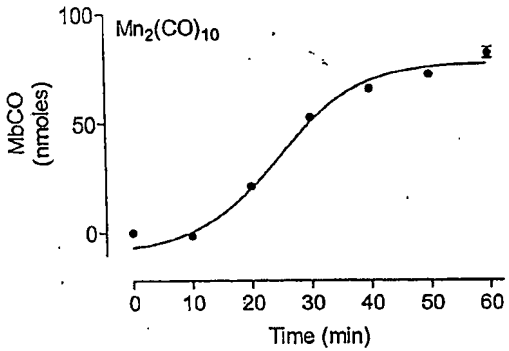


Fig. 2c



Time (min)	MbCO (nmoles)
10	0.3
15	8.3
20	20.5
25	35.7
30	50.4
35	61.7
40	68.9

Fig. 2d

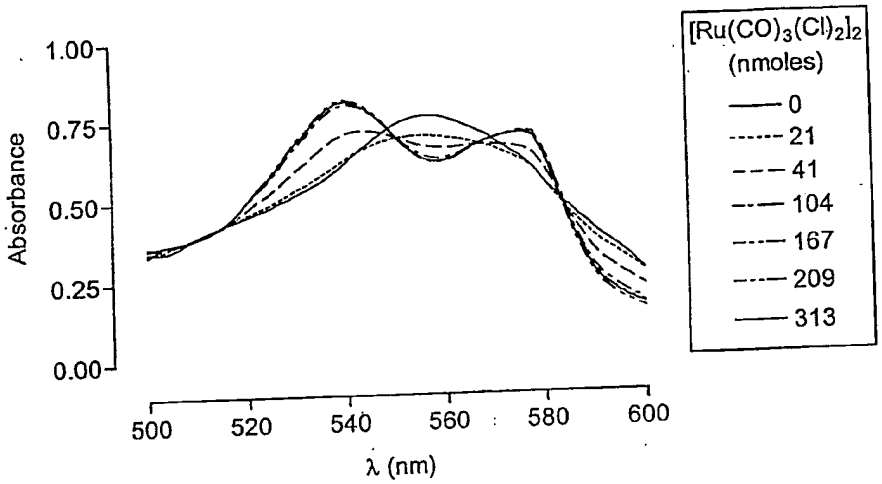

Absorbance
1.00
0.75
0.50
0.25
0.00
500
520
540
560
580
600
λ (nm)
[Ru(CO)3(Cl)2]2
(nmoles)
0
21
41
104
167
209
313

Fig. 2e

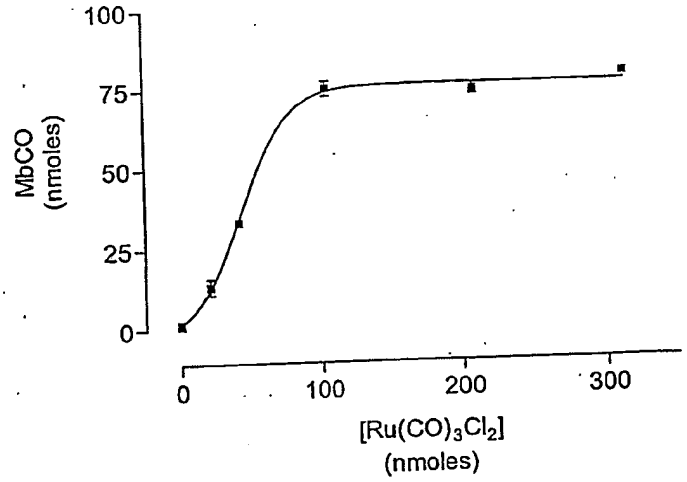
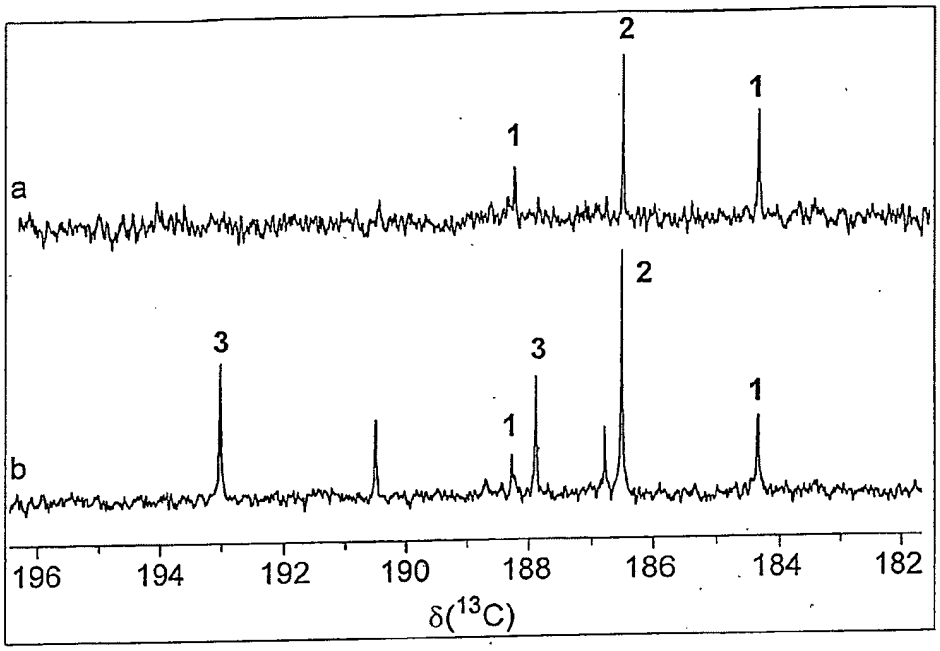
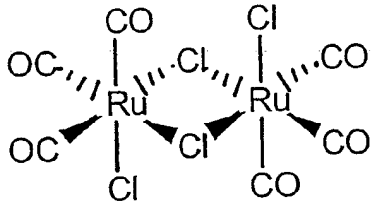
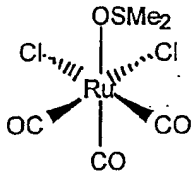

MbCO
(nmoles)
100
75
50
25
0
0
100
200
300
[Ru(CO)3Cl2]
(nmoles)

Fig. 2f

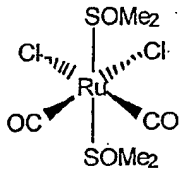




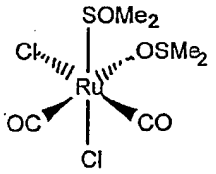
Tricarbonyldichloro ruthenium(II) dimer
$[Ru(CO)_3(Cl)_2]_2$



1



2



3

Fig. 3

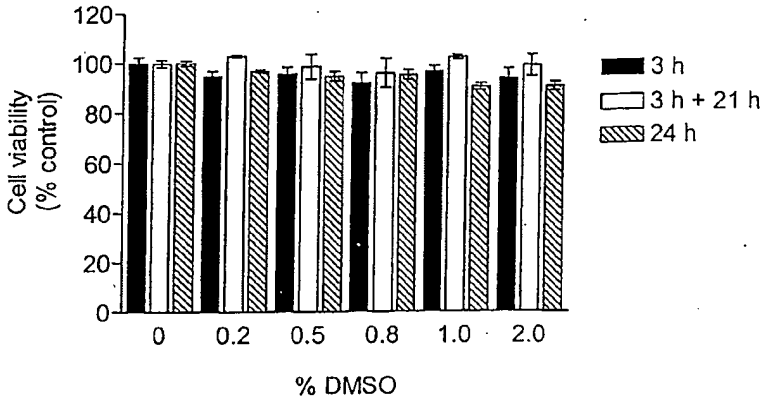


Fig. 4a

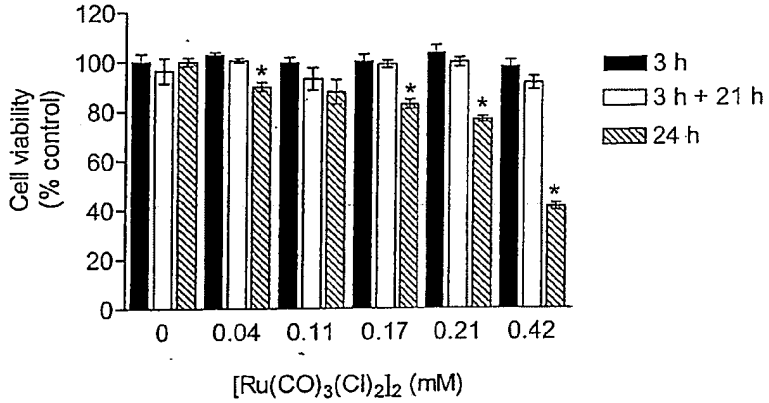


Fig. 4b

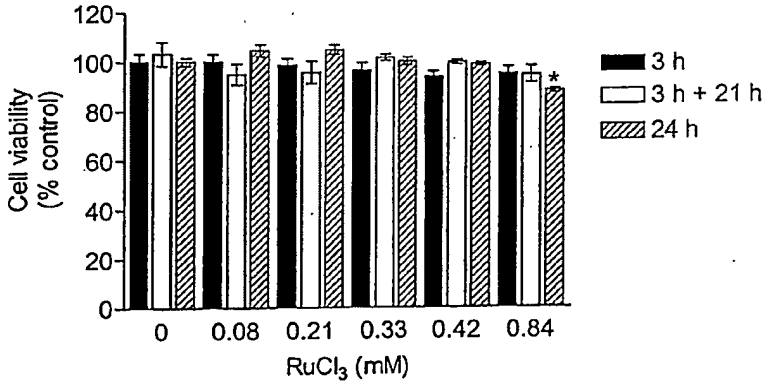


Fig. 4c

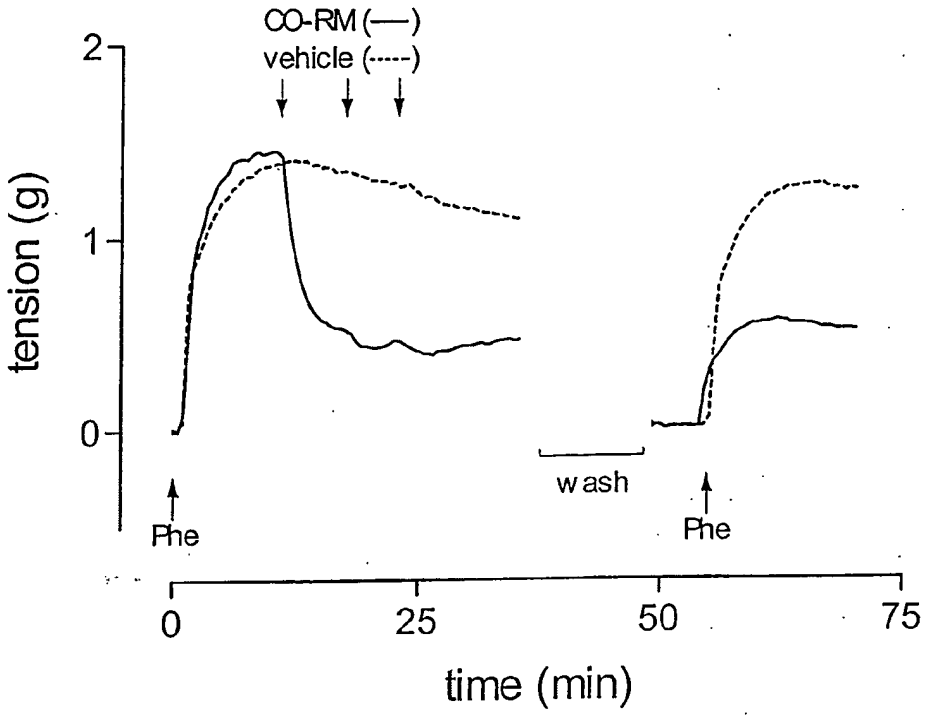

CO-RM (—)
vehicle (----)
2
1
0
tension (g)
Phe
wash
Phe
0
25
50
75
time (min)

Fig. 5

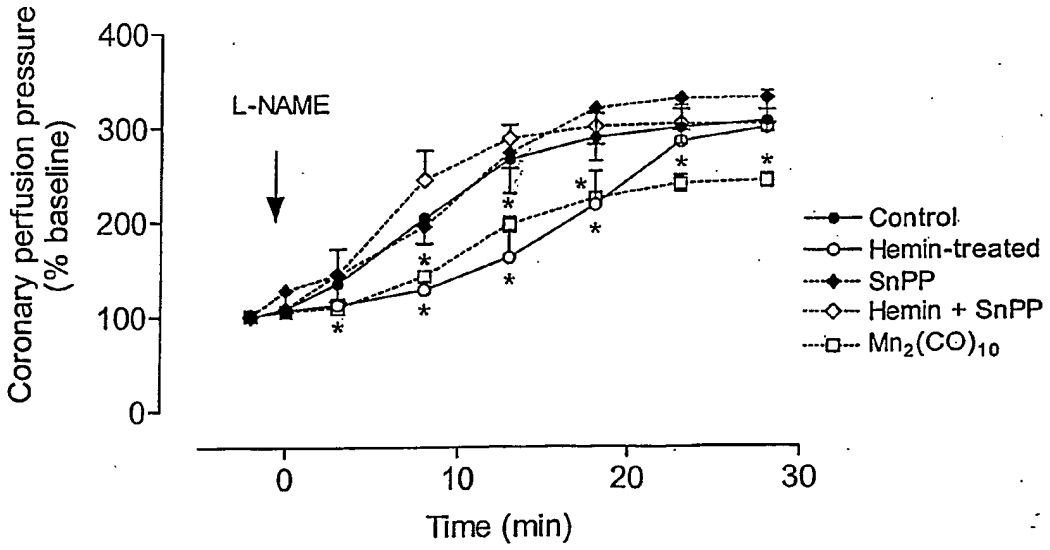

Coronary perfusion pressure
(% baseline)
400
300
200
100
0
L-NAME
0
10
20
30
Time (min)
Control
Hemin-treated
SnPP
Hemin + SnPP
Mn2(CO)10

Fig. 6

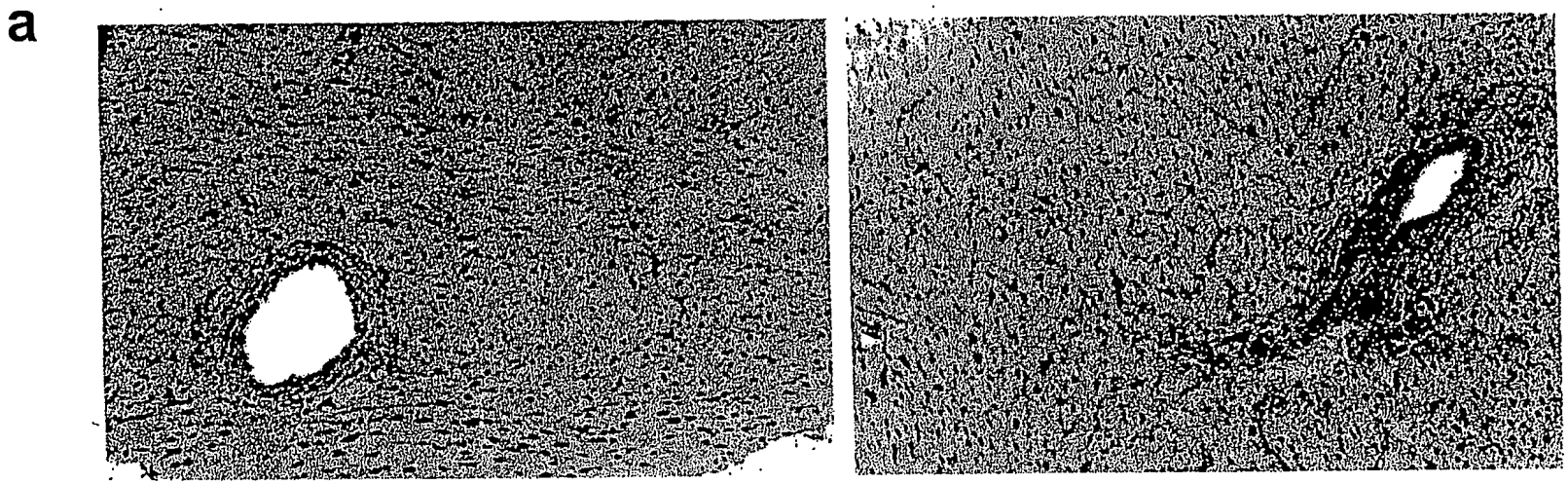


Fig. 7

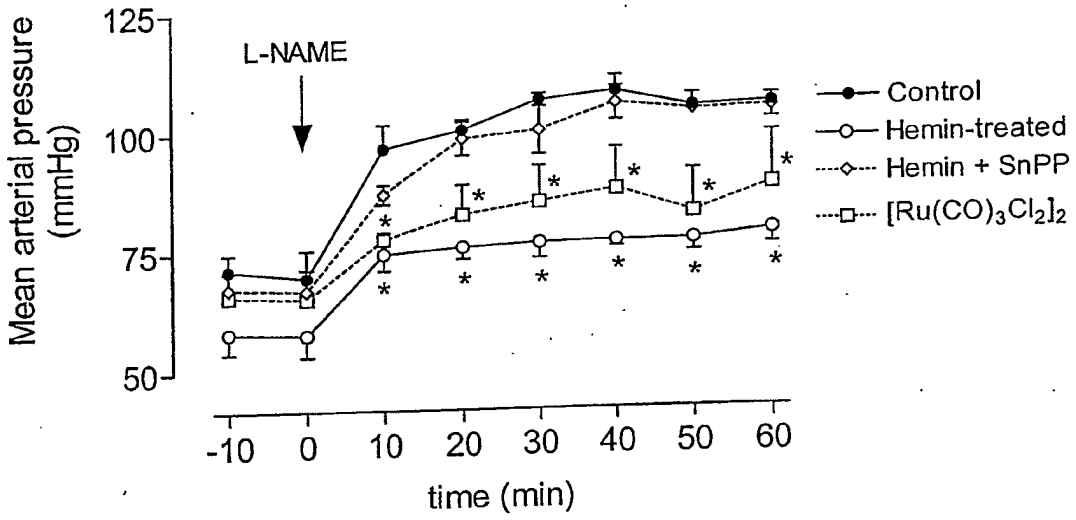

Mean arterial pressure (mmHg)
125
100
75
50
L-NAME
-10
0
10
20
30
40
50
60
time (min)
Control
Hemin-treated
Hemin + SnPP
[Ru(CO)3Cl2]2

Fig. 8